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T-Cell Recognition of a Cross-Reactive Antigen(s) in Erythrocytic Stages of *Plasmodium falciparum* and *Plasmodium yoelii*: Inhibition of Parasitemia by This Antigen(s)

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In the current study, we investigated the presence of a cross-reactive antigen(s) in the erythrocyte stage from *Plasmodium yoelii* (265 BY strain) and *Plasmodium falciparum* through recognition by T cells primed in vivo with antigens from each of these parasites. BALB/c mice are naturally resistant to *P. falciparum* but are susceptible to *P. yoelii* infection. Mice that had recovered from *P. yoelii* primary infection became resistant to a second infection. A higher in vitro proliferative response to a soluble blood stage preparation of *P. falciparum* was observed in splenic cells from immune animals than in those from mice with a patent *P. yoelii* infection. The antigen-induced proliferative response was enhanced when animals were exposed to a secondary infection. Animals exposed to a challenge infection were treated with anti-CD4 or anti-CD8 monoclonal antibodies to deplete the corresponding subset of T cells. There was a marked diminution in *P. falciparum* antigen-induced proliferative response in the total splenic cell populations from CD8-depleted but not from CD4-depleted mice. In CD8-depleted and nondepleted animals, the antigen-induced proliferation in the total cell populations was markedly lower than in the T-cell-rich populations, indicating inhibitory activities of B cells and/or macrophages. There was no such difference in the stimulation between total and T-enriched cell populations from CD4-depleted animals. Flow cytometry analysis demonstrated the presence of an almost equal percentage of $CD8^+$ (59.6%) and $CD4^+$ (64%) T cells in the spleen preparations following in vivo depletion of CD4- and CD8-bearing T cells, respectively. When cultured with *P. yoelii* blood stage antigen, splenocytes from animals immunized with *P. falciparum* antigen displayed a significant proliferative response which was markedly diminished by treatment with anti-Thy-1.2 antibody plus complement. Animals immunized with *P. falciparum* antigen and then challenged with *P. yoelii* blood stage parasites displayed about a 50% lower level of parasitemia. These results demonstrated the existence of a cross-reactive antigen(s) between a murine and a human *Plasmodium* species, as determined from both in vivo and in vitro biological assays, and indicated the reactivity of mainly $CD8^+$ T cells with this antigen.

The life cycle of the parasite that causes malaria, consisting of several stages, provides a number of potential targets for vaccination. Any immunoprophylaxis directed to the erythrocytic stage (blood stage), or merozoite, would control parasite numbers after their release from the liver, preventing or reducing malaria-related morbidity and mortality. Cell-mediated immunity to the blood stage antigen may be important for protection (1, 3, 18). Crisis forms of *Plasmodium falciparum* have been observed in human blood (dying parasites inside erythrocytes are referred to as crisis forms) (20, 27). Supernatants from malaria antigen-activated T cells can induce adherent mononuclear cells to kill parasites in vitro in the absence of antibodies (3). The mechanism of parasite killing was intraerythrocytic (crisis). None of these observations discount an important role for antibodies in human malaria immunity, but they demonstrate the importance of taking into account cellular mechanisms of immunity in vaccine development.

One of the difficulties associated with the development of a successful vaccine is parasite variability, which might result in the avoidance of immunity to *P. falciparum* (2, 4, 21). Although there are a few reports of cross-reactive B-cell epitopes between animal malaria and human malaria parasites (8, 19, 34, 37), very little is known about cross-reactive T-cell epitopes between murine malarial species and *P. falciparum*. Conserved epitopes across diverse species that are the targets of protective immunity in human malaria are clearly preferred for vaccine development.

In the present study, we have attempted to use murine malarial antigens for a human vaccine. As a first step, in the present study, we tested for a cross-reactive antigen(s) in the blood stage of *P. yoelii* and *P. falciparum* parasites which could be recognized by T cells and inhibit parasitemia. Indeed, by using antigen-primed T cells from *P. falciparum*-immunized mice and memory T cells from *P. yoelii*-immune animals, we have been able to provide strong evidence for such a cross-reactive antigen(s). In addition, we have used monoclonal antibodies (MAbs) to CD8 and CD4 to eliminate T-cell subsets in mice immune to *P. yoelii* in order to determine which subset of T cells is reactive to the cross-reactive *P. falciparum* antigen(s).

MATERIALS AND METHODS

Mice. Female BALB/c mice 6 to 8 weeks old were purchased from Iffa Credo, Domaine des Oncins, L'Arbresle, France.

Parasite and infection. The human malarial parasite *P. falciparum* FCR-3 was grown in O-positive human erythro-

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cytes (RBC) supplemented with RPMI 1640 medium (Boehringer, Mannheim, Germany) containing 10% A-positive human serum as described previously (35). The level of parasite growth was 10× per 48-h cycle. Strain FCR-3 was isolated originally in The Gambia.

The 265BY strain of *P. yoelii* was obtained from I. Landau, Natural History Museum, Paris, France. The 265BY strain used in the present study was not a cloned one. Strain 265BY causes a pattern of self-limiting infection in mice similar to that caused by strain 17XNL (22, 25). Aliquots of parasite-carrying RBC (PRBC) were stored in liquid nitrogen. Parasites were maintained in vivo by weekly passage in BALB/c mice. All experiments were performed with animals exposed to parasites which had undergone three in vivo passages. Parasitized RBC were suspended in RPMI 1640, adjusted to the desired concentration, and injected intraperitoneally into passage or experimental mice. A dose of 10^6 PRBC was used for passage mice as well as for experimental mice. The level of parasitemia was determined by counting the percentage of PRBC per 500 RBC on Giemsa-stained thin blood smears. When the parasitemia became less than 1%, at least 5,000 RBC were counted on blood smears. The 265BY strain of *P. yoelii* produced a short-lived parasitemia which often disappeared by day 25 after infection. A few parasites might still be present in the peripheral blood and/or in other organs which were not detected by our blood smear examination. For this reason, the mice were left for another 10 days to clear the residual parasites before exposure to a second infection.

Antigen preparations. (i) *P. yoelii* antigen. When the parasitemia was in an ascending pattern but had not yet reached its peak (usually 30 to 37% parasitemia between days 7 and 12 after inoculation), the mice were bled under anesthesia with diethyl oxide (Merck). Blood was obtained via the carotid artery and pooled in heparinized tubes containing 50 mM phosphate-buffered saline (PBS; pH 7.2). The blood was then passed through a column of glass beads to eliminate leukocytes. The RBC were washed two times with PBS by centrifugation. The RBC were lysed by treatment with 0.06% Saponin (Merck) in PBS by incubation at room temperature for 10 min. After completion of RBC lysis, the liberated parasites were washed twice with PBS, and the pellet was frozen at −20°C. The parasites were then lysed through three cycles of freezing (2 h at −80°C) and thawing (30 min at room temperature). This was followed by disruption (twice for 30 s each) with an ultrasonic probe at 80 W on ice (Sonics & Materials, Inc., Danbury, Conn.). At this stage, mostly liberated parasites and very few nonlysed parasites were observed under the microscope. Nonsoluble material was then separated by centrifugation at 12,000 rpm for 30 min. The quantity of protein in soluble antigenic material was determined with a kit (Bio-Rad, Richmond, Calif.).

(ii) *P. falciparum* antigen. RBC were obtained when 10 to 16% of them in a culture were infected with *P. falciparum*. They were washed twice with PBS by centrifugation. The pellet was then lysed in 0.06% Saponin and subjected to the same protocols used for the preparation of *P. yoelii* antigen. Control extracts from normal O-positive human RBC were prepared in the same way as described for the *P. falciparum* antigenic preparation. The protein content in the soluble antigenic material was then determined with the Bio-Rad kit.

Immunization. For priming, recipient mice were injected subcutaneously with *P. falciparum* blood stage antigen. Control mice were injected with lysed materials from human RBC. In each experiment, age-matched nonimmunized mice were included as controls. For the first injection, antigen was emulsified in Freund's complete adjuvant (Sigma, St. Louis, Mo.) and given subcutaneously at 100 μg per mouse. Subsequently, each mouse received three injections 1 week apart of antigen (70 μg per injection) in Freund's incomplete adjuvant (Sigma). Splenocytes from immunized animals were obtained between 12 and 17 days after the last injection and tested for a proliferative response to homologous and heterologous antigen preparations.

Antibodies. Mouse anti-Thy-1.2 antibody and rabbit complement (Low-Tox-M rabbit complement) were purchased from Cedarlane. Antibodies used for in vivo depletion were GK1.5, a rat immunoglobulin G2b (IgG2b) anti-CD4 antibody (7), and 19/178, a mouse IgG2a anti-CD8 (anti-Ly2.2) antibody (9). Anti-CD8 MAb was kindly provided by Kaj Saizawa (Max-Planck-Institut für Immunbiologie, Freiburg, Germany), and the hybridoma for GK1.5 came from Sakhina Haque (Institut Pasteur, Lille, France). Control antibody for the anti-CD8 antibody was a mouse IgG MAb raised against the conoid protein of *Toxoplasma gondii* and was kindly provided by J.-F. Dubremetz of the Institut National de la Santé et de la Recherche Médicale, Unité 42, Villeneuve d'Ascq, France. The control for anti-CD4 antibody was normal rat IgG prepared from rat serum. Igs were prepared by 50% saturated ammonium sulfate precipitation followed by dialysis against PBS. Immunoglobulin concentrations were determined with a Bio-Rad kit.

In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocytes. Groups of five mice were injected intraperitoneally with GK1.5 (anti-L3T4) or 19/178 (anti-Ly2.2). Treatment of mice with anti-CD4 or anti-CD8 antibody began on day 30 after primary infection, when they were negative for parasitemia. Each mouse was injected with 100 μg of GK1.5 or with 25 μg of 19/178 every day for 10 days and then on alternate days for another 7 days. The mice were challenged with a second infection at day 5 after the treatment with MAbs was begun. The level of depletion of the appropriate T-cell subset was about 90%, as determined by fluorescence-activated cell sorting (FACS) analysis.

Spleen cell fractionation. Spleens were aseptically removed and perfused with 10 ml of Hanks' balanced salt solution. Cell suspensions were centrifuged at 350 × *g* for 10 min. Membrane debris was removed by filtering the cell suspension through sterile gauze. RBC were lysed with lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.11 mM EDTA. To prepare a T-cell-rich population, B cells were removed from the suspension by "panning." Culture plates (60 by 15; Nunc) were coated with 3 ml of a 100-μg/ml preparation of goat anti-mouse Ig in PBS and incubated overnight at 4°C. Splenocytes, at a maximum of 18×10^6 per plate in 3 ml of Hanks' balanced salt solution containing 5% fetal calf serum, were incubated for 1 h at room temperature. Nonadherent cells were obtained by gently washing the plates with medium, and after the cells had been adjusted to the original concentration, the panning procedure was repeated. The T-cell-enriched population thus obtained ranged between 84 and 90% T cells, as determined by FACS analysis with anti-Thy1.2 antibody (data not shown).

T-cell depletion of splenocyte populations. T cells were depleted from unfractionated splenocytes by incubating 10×10^6 cells at 4°C for 1 h with 30 μl of anti-Thy1.2 antibody plus 0.7 ml of Hanks' solution containing 5% fetal calf serum. The cells were then centrifuged, and the pellet was resuspended in 0.7 ml of Hanks' solution containing 5% fetal calf serum and complement (Low-Tox-M rabbit complement; Cedarlane) at a 1:10 dilution. After incubation for 1 h at 37°C in a

humidified atmosphere, dead cells were removed by centrifugation on a density gradient (Lymphoprep; Nicomed AS, Oslo, Norway) and washed two times in Hanks' balanced salt solution plus 5% fetal calf serum. The viability of both unfractionated and fractionated populations was regularly determined by trypan blue dye exclusion and was greater than 90%.

Lymphocyte proliferation assay. Spleen cells were prepared as described above. Cultures were set up at a concentration of 2.5×10^5 cells per well in 96-well plates (Nunc) in complete medium (RPMI 1640 plus 10% heat-inactivated fetal calf serum, 25 mM HEPES buffer [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 2 mM L-glutamine, 10^{-5} M 2-mercaptoethanol, and 50 μg of gentamicin per ml). A volume of 0.05 ml of medium alone or medium containing 20 μg of *P. falciparum* blood stage soluble antigen or control antigen per ml in 0.150 ml of medium was added to each well which had aliquots of cells. The cells were cultured for 5 days in the presence of parasite or control antigen. The doses of antigens and the duration of culture for optimal stimulation were determined in preliminary experiments. For the last 16 h of culture, 0.5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 74 GBq/mmol; 2 Ci/mmol; Amersham) was added to each well. Cells were harvested with an automatic cell harvester (Skatron Instruments AS). Incorporated radioactivity was measured in a liquid scintillation counter (LS-1800 Beckman scintillation counter). The degree of lymphocyte proliferation is expressed by the stimulation index (SI): SI = average cpm in stimulated cultures/average cpm in unstimulated cultures. The interassay SI variations were within 10%. Mean SIs ± standard deviations were used to test significance.

FACS analysis. FACS analysis was performed essentially as described elsewhere (15). Aliquots of 10^6 cells that did not adhere to anti-mouse Ig-coated plates were transferred to microtubes. The cells were incubated in 100 μl of 50 mM PBS (pH 7.2) supplemented with 5% fetal calf serum (Serovial) containing 100 μg of either anti-Thy1.2, anti-CD4, or anti-CD8 MAb per ml. The fluorescein conjugate used was goat anti-rat IgG (heavy and light chain; Sigma) when cells were incubated with GK1.5 MAb or goat anti-mouse IgG (Tago, Inc., Burlingame, Calif.) when cells were treated with 19/178 MAb. The fluorescein-labeled antibodies were used at a dilution of 1:50. All incubations were carried out on ice. Labeled cells were analyzed by flow cytometry (Epics Profile; Coulter Electronics, Inc.).

Statistical analysis. Data were analyzed for differences by the Student *t* test. A probability of less than 0.05 was considered significant.

RESULTS

Spleen cells from *P. yoelii*-immune animals proliferated significantly in the presence of *P. falciparum* blood stage antigen. BALB/c mice were infected with *P. yoelii*, and splenocytes from these animals were tested at different times after infection for their ability to proliferate in response to a heterologous blood stage soluble antigen preparation from *P. falciparum*. No significant proliferation was induced by *P. falciparum* antigen as long as the erythrocytic stage of *P. yoelii* persisted in the circulation. When the parasitemia disappeared in the primary infection, there was a marked increase in the stimulatory response of spleen cells from *P. yoelii*-infected mice to *P. falciparum* antigen (SI, 4.2 ± 0.38). The proliferative response of splenocytes to *P. falciparum* antigen was augmented further when animals were challenged with a second infection at day 35 after the first infection and assayed 15 days later (SI, 9.2 ± 0.94). The response of mice which were not challenged at day 50 did not change significantly from that at day 35 (SI, 3.7 ± 0.14). Cells from age-matched normal mice did not show any significant response when stimulated with the *P. falciparum* blood stage preparation. There was no significant proliferative response of cells obtained at any time after primary or secondary infection towards a control preparation from uninfected human RBC. These results are summarized in Fig. 1.

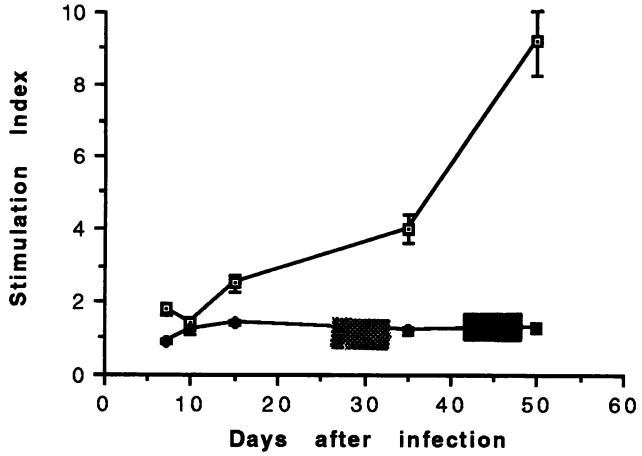

FIG. 1. In vitro proliferative response of splenocytes from *P. yoelii*-immune mice in the presence of *P. falciparum* blood stage antigen. Antigen-induced reactivity of spleen cells was determined during the patent and postpatent periods of infection. At day 35 postinfection, animals were challenged with a second infection, and lymphocyte proliferation was measured on day 15 after reinfection. *P. falciparum* antigen (●, □) or human RBC lysate (shaded and solid rectangles) (20 μg/ml) was used for stimulation. Spleen cells were from age-matched, noninfected controls (● and shaded rectangle) or from *P. yoelii*-infected mice (□ and solid rectangle). The proliferative response of spleen cells from 35 days postinfection to *P. falciparum* antigen (5,743 ± 832 cpm) was significantly higher than that to the human RBC preparation (1,821 ± 133 cpm) ($P < 0.05$). The response of splenocytes obtained from animals at day 15 after reinfection was significantly higher to *P. falciparum* antigen (14,007 ± 1,222 cpm) than to RBC extract (2,208 ± 1,542 cpm) ($P < 0.05$). Cells from age-matched normal mice did not show marked proliferation in response to *P. falciparum* antigen (1,725 ± 125 cpm). The data are representative of five experiments.

Splenocytes from animals immunized with soluble blood stage antigen of *P. falciparum* parasites stimulated significantly when cultured with *P. yoelii* antigen. Two groups of animals were immunized with a soluble antigen preparation from blood stage parasites of *P. falciparum* or with a preparation of lysed human RBC (RBC used for the *P. falciparum* culture). Splenocytes from these two groups as well as from nonimmunized control animals were assayed for proliferative response to either *P. falciparum* antigen or *P. yoelii* blood stage antigen. There was significant stimulation in response to the *P. falciparum* antigen by splenocytes obtained from *P. falciparum* antigen-immunized animals but not by spleen cells from human RBC-immunized or nonimmunized control animals (Fig. 2a). Interestingly, although it was less marked, there was a significant proliferative response to heterologous *P. yoelii* blood stage antigen by splenocytes from *P. falciparum*-immunized mice but not by

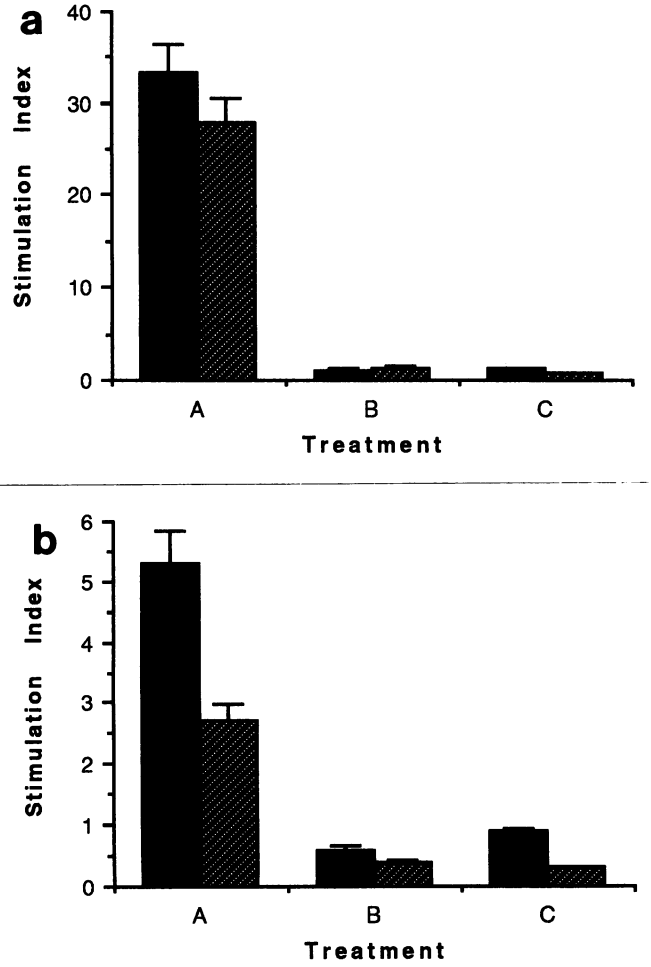

FIG. 2. Proliferation of splenocytes obtained from animals at day 17 after immunization with *P. falciparum* antigen in response to *P. yoelii* antigen. BALB/c mice were immunized with blood stage *P. falciparum* antigen (A) or with lysed human RBC (B); control animals were not immunized (C). SIs are shown for *P. falciparum* (a) and *P. yoelii* (b) blood stage antigen at 20 μg/ml (solid bars) and 10 μg/ml (hatched bars). The levels of the proliferative response of the unstimulated cells were 947 ± 263, 754 ± 163, and 789 ± 217 cpm for A, B, and C, respectively. The data are from one representative experiment. Interassay variation was within 10%.

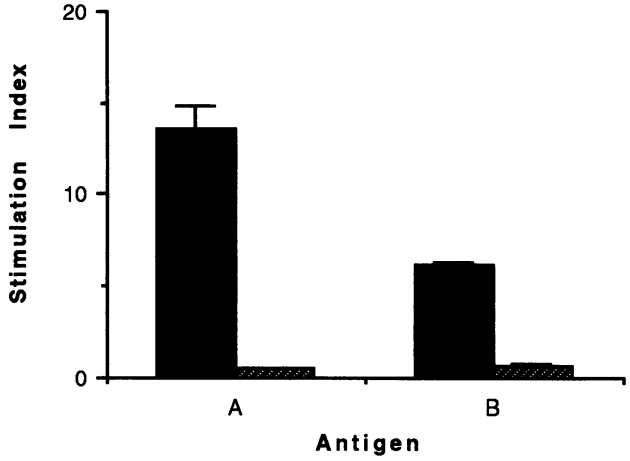

FIG. 3. Effect of treatment of splenocytes from immunized animals with anti-Thy1.2 plus complement on proliferation in the presence of *P. falciparum* or *P. yoelii* antigen. Spleen cells were obtained on day 12 after the last immunization with *P. falciparum* antigen, and their stimulation by *P. falciparum* (A) and *P. yoelii* (B) blood stage antigen was determined before T-cell depletion (solid bars) and after T-cell depletion by anti-Thy1.2 treatment (hatched bars). The unstimulated cells gave values of 5,554 ± 3,190 and 1,819 ± 779 cpm for A and B, respectively. This experiment was repeated three times.

those from lysed human RBC-immunized or nonimmunized control animals (Fig. 2b).

In vitro depletion of T lymphocytes in a spleen cell population obtained from *P. falciparum* antigen-immunized animals resulted in a decrease in antigen-induced proliferation. Splenocytes from *P. falciparum*-immunized animals were treated with anti-Thy1.2 antibody plus rabbit complement to deplete T cells (see Materials and Methods) and assayed for their response to *P. falciparum* and *P. yoelii* blood stage antigen. The proliferative responses of T-cell-depleted splenocytes to both antigens were dramatically reduced (Fig. 3).

Effects of depletion of CD4 or CD8 T cells in *P. yoelii*-immune animals on lymphocyte proliferation to *P. falciparum* blood stage antigen. After recovery from *P. yoelii* primary infection, mice were treated with anti-CD4 or anti-CD8 MAb starting at day 30 postinfection. Daily injections of these MAbs were continued for 5 days, and the animals were then exposed to a secondary infection. Injections of the anti-T-cell subset MAbs continued for another 12 days. At day 15 after reinfection, splenocytes were obtained from these animals and assayed for blastogenesis in the presence of parasite antigen. A marked decrease in the proliferative responses of total cell populations from CD8-depleted but not from CD4-depleted mice to *P. falciparum* antigen was observed. A different pattern in antigen-induced stimulation emerged when T-cell-rich preparations were used in the assay. In nondepleted as well as in CD8-depleted cell samples, the proliferative response to the *P. falciparum* antigen preparation by T-cell-rich populations was significantly higher than by total cell populations. In contrast, there was no such difference in stimulation when total and T-cell-rich populations from CD4-depleted animals were tested. The lymphocyte proliferative response to the *P. falciparum* antigen preparation by T-cell-rich populations was significantly diminished in CD4- or CD8-depleted mice compared with that in intact animals. These results are shown in Fig. 4.

Phenotypic analysis of T-cell subset by FACS after in vivo depletion. Spleen cells were obtained from both CD4- and CD8-depleted animals at day 15 after reinfection and subjected to FACS analysis. The cells obtained for FACS did not adhere to anti-Ig-coated plates. The percentages of $CD4^+$ and $CD8^+$ T cells present in intact mice were 45.9 and 15%, respectively. When mice received injections of anti-CD8 MAb, the percentage of $CD8^+$ cells dropped to 3.1% with a background value of 1.7%, whereas the percentage of $CD4^+$ T cells rose to 64.1%. In animals that received injections of anti-CD4 MAb, the percentage of $CD4^+$ T cells diminished to 3.4% with a background value of 2.8%, and the percentage of $CD8^+$ T cells rose to 59.6%. These results are presented in Table 1.

Immunization with soluble blood stage antigen of *P. falciparum* resulted in diminished parasitemia in *P. yoelii*-infected BALB/c mice. Groups of five mice were immunized with *P. falciparum* antigen or with human RBC extract by four subcutaneous injections as described in Materials and Methods. Six days after the last injection, the immunized animals

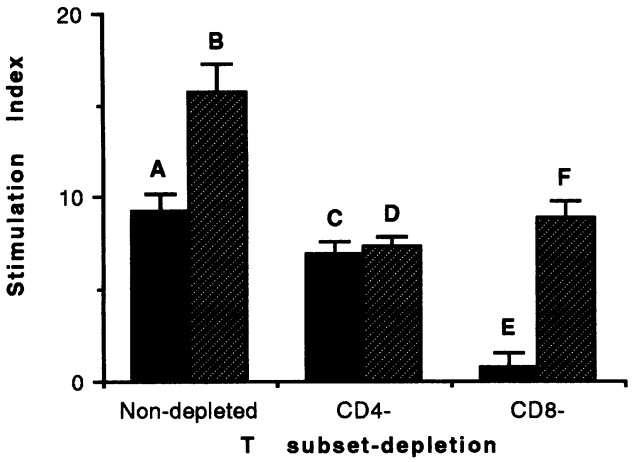

FIG. 4. Effect of depletion of CD4- or CD8-bearing T cells in *P. yoelii*-reinfected animals on *P. falciparum* antigen-induced lymphocyte proliferation. There was a marked decrease in proliferative response when the total cell population from CD8-depleted animals was tested. SIs are shown for total cell (solid bars) and T-cell-rich (hatched bars) populations. The levels of the proliferative response of the unstimulated total cells were 992 ± 527, 858 ± 405, and 2,808 ± 559 cpm for nondepleted, CD4-depleted, and CD8-depleted populations, respectively. The values for unstimulated T-cell-rich populations were 1,981 ± 170, 668 ± 277, and 864 ± 182 for nondepleted, CD4-depleted, and CD8-depleted populations, respectively. Comparisons were made with the Student *t* test, taking into account the means and standard deviations of several experiments. A versus C, $P > 0.05$; A versus E, $P < 0.05$; B versus D, $P < 0.05$; B versus F, $P < 0.05$; A versus B, $P < 0.05$; C versus D, $P > 0.05$; E versus F, $P < 0.05$.

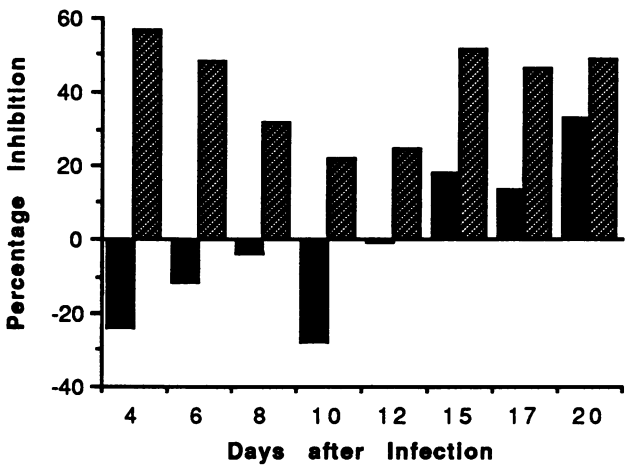

FIG. 5. Immunization of mice with *P. falciparum* antigen resulted in decreased parasitemia during *P. yoelii* infection. The decrease in parasitemia (percentage of inhibition) was determined for animals immunized with *P. falciparum* blood stage antigen (hatched bars) or injected with control human RBC lysate (solid bars). The data represent the results of one typical experiment of three experiments. The percent decrease was determined by the following formula: 100 − [(parasitemia in antigen-treated mice/parasitemia in nontreated infected mice) × 100].

were challenged with one million *P. yoelii* PRBC. On days 4 and 6 postinfection, there was 56.6 and 48.4% reduction, respectively, in parasitemia in *P. falciparum* antigen-immunized animals, whereas no such decrease in parasitemia was observed in mice injected with control extract. The decrease was less marked on days 10 and 12 after infection. The percent decrease increased again to 52% at day 15 and remained high until day 20 postinfection. The parasitemia decreased rapidly thereafter, disappearing completely in both control and immunized animals by day 25 after infection. These results are shown in Fig. 5.

DISCUSSION

In the present study, we investigated the presence of cross-reactive T-cell determinants in blood stage antigen preparations between *P. yoelii*, which is a murine malarial parasite, and *P. falciparum*, which is a human malarial parasite. Our objective was to identify a T-cell epitope(s) which is conserved between these distantly related species and is not polymorphic. The role of this antigen in protection or pathophysiology in malarial infections will then be examined. This approach appears to be particularly relevant when one considers the problems of parasite variability and the poor level of immunity induced by a clinical attack. At this stage of our study, we decided to work not with peptides but with the native proteins because subsequent fractionation studies should lead to appropriate mapping of functional epitopes which might be missed if peptides were used at the outset. It has recently been shown for *P. falciparum* that recombinant proteins representing amino acid residues 147 to 321 of p190, or residues 1060 to 1195 of p190-3, when used in protection experiments in monkeys, did not elicit the protection conferred by native gp190 (10). In addition, we do not yet know the frequency of antigen-reacting T cells. A lower proliferative response in malaria-infected individuals to purified antigen or peptides than to whole-parasite lysate was reported (5).

TABLE 1. Phenotypic analysis of spleen cells after in vivo depletion of T-cell subsets[a]

MAb	Fluorescence (% of cells)			
	Untreated	Control MAb	Anti-CD4 MAb	Anti-CD8 MAb
Control anti-rat Ig	1.3	1.5	2.3	0.8
Anti-CD4	45.9	37.3	3.4	64.1
Anti-CD8	15.0	14.7	59.6	3.1
Anti-Thy1.2	88.2	90.1	85.2	91.1
Control anti-mouse Ig	2.2	2.4	2.8	1.7

[a] Animals treated with anti-CD4 or anti-CD8 MAb were killed on day 15 after secondary infection, and their spleen cells were analyzed by FACS. Values show the fluorescence of 10,000 live cells in each preparation.

The results of our study demonstrate that spleen cells obtained from mice immunized with the soluble *P. falciparum* blood stage antigen preparation responded to both homologous *P. falciparum* and heterologous *P. yoelii* antigen, as measured by in vitro proliferation. The reactive cells were T cells, because depletion of T cells by treatment with anti-Thy1.2 antibody plus complement abrogated this antigen-induced proliferation.

Spleen cells obtained at day 35 postinfection from mice which had recovered from *P. yoelii* primary infection displayed a significant proliferative response to the *P. falciparum* blood stage antigen preparation. The response was markedly enhanced when animals were challenged with a second infection at day 35 after the first infection and the lymphoproliferative assay was done 15 days later. The response of mice which were not challenged at day 50 did not

change significantly from that at day 35. This suggests that an increase of 15 days in the age of the mice has no effect on their response to the antigen. Our results showed that repeated infections could lead to increased T-cell reactivity to the parasite antigen. The enhanced antigen reactivity of T cells may be related to a larger pool of memory T cells in the secondary infection than in the primary infection. In humans, immunity to malaria develops with age after repeated exposure to parasite infections (12, 26). The lymphoproliferative response to the *P. falciparum* antigen was depressed during patent *P. yoelii* infection. Similar cellular unresponsiveness to specific antigen and T-cell mitogens was reported in several malarial infections (14, 23a, 36, 39). The mechanism underlying such inactivation ("anergy") of memory cells during the course of malarial infections remains unclear.

The soluble blood stage antigen preparation from *P. falciparum* used in our experiment did not appear to be mitogenic for normal BALB/c spleen cells because they did not proliferate in the presence of different concentrations of antigen in our assay system. Moreover, proliferative response towards *P. falciparum* antigen was not significant with spleen cells from animals parasitemia. Such a differential proliferative response would not be based on the presence of mitogenic, as opposed to antigenic, material in *P. falciparum*-infected RBC. Previous reports of mitogenic activity in supernatants or lysates of malaria-infected RBC have been based on the proliferative response of cells from normal, noninfected individuals to *P. falciparum* extracts (13, 28, 40). However, others failed to observe such mitogenic activity (29, 36). Furthermore, the failure of spleen cells to proliferate in the presence of a control preparation of normal human RBC indicates the absence of any material in them mitogenic for mouse lymphocytes.

The results of our study strongly suggest the presence of a cross-reactive T-cell antigen(s) between *P. yoelii* and *P. falciparum*. It was recently shown that some human $CD4^+$ T cell clones from *P. falciparum*-infected individuals cross-react with the mouse parasite *Plasmodium berghei* (29).

Taking advantage of the *P. yoelii*-mouse system, we have investigated what subset of T cells was reactive to *P. falciparum* antigen. For this purpose, $CD4^+$ and $CD8^+$ T cells were depleted in vivo by treatment of mice with MAbs to these subsets. Unfractionated and T-cell-rich spleen cell populations were assayed for their proliferative response to soluble *P. falciparum* blood stage antigen. The *P. falciparum* antigen-induced proliferative response was markedly decreased in total spleen cells from CD8-depleted but not from CD4-depleted mice. These results suggest that cell populations composed mainly of CD8-bearing T cells were reactive to *P. falciparum* antigen in *P. yoelii*-immune animals. The potential protective role of $CD8^+$ T cells that react to sporozoite antigen was suggested for murine as well as human malarial infections (11, 24, 32, 38). Very little is known about the role of $CD8^+$ T cells in immunity to the erythrocytic stage in malarial infections. $CD8^+$ T cells that react to the cross-reactive antigen may well participate in the clearance of parasites from the circulation via lymphokine-dependent and macrophage-mediated antiparasite activities (6, 17, 27, 30, 33).

The results of our study demonstrated that T-cell-rich populations from CD8-depleted and intact mice responded better to *P. yoelii* antigen than the total cell population. One possible explanation for this improved response may be the presence of a higher percentage of antigen-reactive T cells in the enriched populations. The diminished response by the total cell populations might also relate to suppressive activities on antigen-driven T-cell expansion by B cells and/or macrophages which were removed during panning. Suppressive functions of macrophages in malarial infections were suggested previously (14, 18, 23). The inhibitory role of B cells or their factors in two other parasitic infections was reported (16, 31). The diminished response observed in spleen cells from CD8-depleted mice was not related to the difference in the number of T cells tested because an equal number of cells were assayed whether they were obtained from CD8-depleted or CD4-depleted animals. In addition, the results of the FACS analysis indicated almost equal numbers of T cells in CD4-depleted (64%) and CD8-depleted (59.6%) mice. Although a few antigen-presenting cells might be removed from the splenocytes when they are subjected to panning, enough of them would be left in the population to effect antigen-processing and costimulatory activities, since the percentage of T cells in T-cell-enriched populations varied between 84 and 90%.

In preliminary experiments, the protective role of the cross-reactive antigen present in *P. falciparum* was tested by immunizing BALB/c mice with *P. falciparum* blood stage antigen and then exposing them to *P. yoelii* blood stage parasites. The diminution in parasitemia observed varied between 50 and 60% during the critical period of infection. This decrease appears to be important if we take into consideration the challenge dose and the particular host-parasite model used. Even partial immunity to the asexual blood stage parasites could be important, since disease severity is related to the level of parasitemia (12, 20, 26).

In the current study, by using T lymphocytes primed with *P. falciparum* antigens and memory T cells generated in *P. yoelii* infection, we have been able to show clearly the presence of a cross-reactive antigen(s) between *P. yoelii* and *P. falciparum*. In the *P. yoelii*-immune mice, this cross-reactive antigen was recognized by CD8-bearing T cells. In addition, we have produced evidence of the potential protective property of this cross-reactive antigen. To our knowledge, the existence of a cross-reactive antigen(s) in the erythrocytic stage between *P. falciparum* and *P. yoelii* based on both in vivo and in vitro biological assays, and the identification of a T-cell subset reactive to this antigen, was not demonstrated previously. The valuable information gathered in this study will allow us to undertake biochemical isolation and molecular cloning of this antigen and subsequent characterization of the antigen. Further experiments are in progress to study whether similar $CD8^+$ T cells can be generated against this antigen in humans and to determine what role they can play in human malaria.

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